Intact transferrin and total plasma glycoprofiling for diagnosis and therapy monitoring in phosphoglucomutase-I deficiency

NURULAMIN ABU BAKAR, NICOL C. VOERMANS, THORSTEN MARQUARDT, CHRISTIAN THIEL, MIRIAN C.H. JANSSEN, HANA HANSIKOVA, ELLEN CRUSHELL, JOLANTA SYKUT-CEGIELSKA, FRANCIS BOWLING, LARS MØRKRID, JOHN VISSING, EVA MORAVA, MONIQUE VAN SCHERPENZEEL, and DIRK J. LEFEBER

NIJMEGEN, THE NETHERLANDS; MUENSTER, AND HEIDELBERG, GERMANY; PRAGUE, CZECH REPUBLIC; DUBLIN, REPUBLIC OF IRELAND; WARSAW, POLAND; SOUTH BRISBANE, AUSTRALIA; NORWAY; DENMARK; AND ROCHESTER, MINNESOTA

Phosphoglucomutase 1 (*PGM1*) deficiency results in a mixed phenotype of a Glycogen Storage Disorder and a Congenital Disorder of Glycosylation (CDG). Screening for abnormal glycosylation has identified more than 40 patients, manifesting with a broad clinical and biochemical spectrum which complicates diagnosis. Together with the availability of D-galactose as dietary therapy, there is an urgent need for specific glycomarkers for early diagnosis and treatment monitoring. We performed glycomics profiling by high-resolution QTOF mass spectrometry in a series of 19 PGM1-CDG patients, covering a broad range of biochemical and clinical severity. Bioinformatics and statistical analysis were used to select glycomarkers for diagnostics and define glycan-indexes for treatment monitoring. Using 3 transferrin glycobiomarkers, all PGM1-CDG patients were diagnosed with 100% specificity and sensitivity. Total plasma glycoprofiling showed an increase in high mannose glycans and fucosylation, while global galactosylation and sialylation were severely decreased. For treatment monitoring, we defined 3 glycan-indexes, reflecting normal glycosylation, a lack of complete glycans (LOCGI) and of galactose residues (LOGI). These indexes showed improved glycosylation upon D-galactose treatment with a fast and near-normalization of the galactose index (LOGI) in 6 out of 8 patients and a slower normalization of the LOCGI in all patients. Total plasma glycoprofiling showed improvement of the global high mannose glycans, fucosylation, sialylation, and galactosylation status on D-galactose treatment.

From the Department of Neurology and Translational Metabolic Laboratory, Donders Institute for Brain, Cognition, and Behavior, Radboud University Medical Center, Nijmegen, The Netherlands; Department of Neurology, Radboud University Medical Center, Nijmegen, The Netherlands; Uni-University Hospital Muenster, Muenster, Germany; Center for Child and Adolescent Medicine, Kinderheilkunde I, University of Heidelberg, Heidelberg, Germany; Department of Internal Medicine, Radboud University Medical Center, Nijmegen, The Netherlands; Department of Pediatrics and Adolescent Medicine, First Faculty of Medicine, Charles University in Prague and General University Hospital in Prague, Prague, Czech Republic; Academic Centre on Rare Diseases, University College Dublin, Dublin, Republic of Ireland; Department of Inborn Errors of Metabolism and Paediatrics, Institute of Mother and Child, Warsaw, Poland; Biochemical Diseases, Mater Children's Hospital, South Brisbane, Queensland, Australia; Institute of Clinical Biochemistry, Faculty of Medicine, University of Oslo and Department of Medical Biochemistry, Oslo University Hospital, Norway; Department of Neurology, University of Copenhagen, Denmark; Department of Clinical Genomics, CIM, Mayo Clinic, Rochester, Minnesota; Translational Metabolic Laboratory, Radboud University Medical Center, Nijmegen, The Netherlands.

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Reprint requests: Dirk J. Lefeber, Translational Metabolic Laboratory, Department of Neurology, Radboud University Medical Center, Box 9101, 6500 HB Nijmegen, The Netherlands. e-mail: dirk.lefeber@radboudumc.nl.

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Our study indicates specific glycomarkers for diagnosis of mildly and severely affected PGM1-CDG patients, and to monitor the glycan-specific effects of D-galactose therapy. (Translational Research 2018; 199:62–76)

Abbreviations: PGM1 = phosphoglucomutase-1; G1P = glucose-1-phosphate; CDG = congenital disorder of glycosylation; CDG-I = CDG type-1; CDG-II = CDG type-2; MS = mass spectrometry; TIEF = transferrin isoelectric focusing; ApoC-III = apolipoprotein C-III; IEF = isoelectric focusing; v/v = volume/volume; PGC-chip = porous graphitized carbon chip; CI = confidence intervals; NGI = normal glycan index; LOCGI = lack of complete glycan index; LOGI = lack of galactose index; ER = endoplasmic reticulum

At a Glance Commentary

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Background

Early recognition of phosphoglucomutase-I deficiency (PGM1-CDG) is crucial to initiate metabolic interventions. However, diagnosis of PGM1-CDG is highly challenging due to the large clinical and biochemical heterogeneity. We perfomed glycomics profiling by mass spectrometry to identify unique glycomarkers.

Translational Significance

Specific transferrin glycoforms were identified for fast and accurate diagnosis of the full range of clinical presentations, and for monitoring of specific effects of oral D-galactose therapy. These glycomarkers will result in early diagnosis of novel PGM1-CDG patients, and allow further optimization of oral D-galactose as supplementation therapy.

INTRODUCTION

Phosphoglucomutase 1 (PGM1) deficiency was first reported in an adult patient with exercise-induced muscle cramps and several episodes of rhabdomyolysis, designated as glycogen storage disorder type XIV. Phosphoglucomutase 1 is a cytosolic enzyme that interconverts glucose-1-phosphate and glucose-6-phosphate, and is thus essential to generate energy via the glycolytic pathway from glucose-1-phosphate, as released from glycogen upon exercise.[1] In a cohort of unsolved Congenital Disorders of Glycosylation (CDG) patients, *PGM1* mutations (PGM1-CDG) were found in 2 children with multisystem features, including cleft palate, short stature, cardiomyopathy, hypoglycemia, liver function and endocrine abnormalities, and hypoglycemia.[2]

Genetic analysis of additional cohorts of unsolved CDG patients revealed a large group of 19 PGM1-CDG patients, with mixed CDG-type 1 (CDG-I) and CDG-type 2 (CDG-II) screening profiles. The clinical presentation ranged from a predominant liver involvement, early-onset hypoglycaemia to myopathy with or without the multisystem features as described above. Mass spectrometry (MS) of intact transferrin in some patients showed a combination of transferrin isoforms with a lack of glycans, and abnormal glycan structures lacking galactose.[3] The presence of specific clinical features, such as bifid uvula with or without cleft palate at birth, in combination with abnormal protein glycosylation has alerted early suspicion of PGM1-CDG patients, and has indeed made this a fast growing novel CDG group. Dietary D-galactose supplementations in 6 patients were shown to improve the glycosylation profiles and clinical symptoms. Especially, 2 teenage girls exhibited a disappearance of rhabdomyolysis, and normalization of hypogonadotropic hypogonadism leading to clinical signs of puberty.[3] Several clinical trials were established to study the effect of oral D-galactose.[4-6]

In view of the broad clinical spectrum and the variety in CDG screening profiles of PGM1-CDG, the challenge is to identify unique biochemical markers that can unambiguously detect this disorder among the complete spectrum of clinical presentations. In addition, the introduction of D-galactose as dietary intervention necessitates the accurate monitoring of therapy responses. To solve the diagnostic challenge of PGM1-CDG, we performed extensive glycomics profiling of a large group of 19 PGM1-CDG patients, covering the full range of clinical features and including 8 patients on oral D-galactose to find specific glycomarkers for direct diagnosis and treatment monitoring.

MATERIALS AND METHODS

All chemicals and reagents were of the highest level of purity available. Unless otherwise stated, they were purchased from Sigma-Aldrich.

PLASMA SAMPLES

For establishment of reference ranges, plasma samples from a *control* group of 20 healthy volunteers (>18 years, n = 5; 2–18 years, n = 5; 1 month-2 years, n = 5 and <1 month, n = 5) were analyzed for transferrin

isoelectric focusing (TIEF) and QTOF MS to define normal transferrin glycosylation. Plasma samples of all patients were obtained from the diagnostics archive (Radboud UMC, Nijmegen, The Netherlands) and used in agreement with Helsinki's Declaration. Patient samples were from the *case* group of PGM1-CDG (n = 19), and a *contrast* group of mild CDG-I (n = 10), galactosemia due to GALT deficiency (n = 3), B4GALT1-CDG (n = 3), and SLC35A2-CDG (n = 3). The group of mild CDG-I is randomly selected from a cohort of CDG-I patient samples showing a mild increase of CDG-I transferrin glycoforms. The diagnosis of all PGM1-CDG patients was genetically (Table I) and biochemically confirmed (Supplementary Table I).[1,3,5-8] As previously described, a series of P12 samples covering before, during and after 6 months D-galactose therapy were analyzed to define 3 glycan-indexes from intact transferrin glycoprofiling.[5] Moreover, samples from 8 PGM1-CDG patients including P12 (Table I) before and during 3 months of D-galactose therapy were available for therapy monitoring study using the 3 defined glycan-indexes (clinicaltrials.gov NCT02955264).[5,6]

CDG SCREENING TESTS

Routine screening tests for protein N-glycosylation by TIEF as well as for protein mucin type O-glycosylation by apolipoprotein C-III isoelectic focusing (IEF) were performed as described.[9]

HPLC-CHIP-QTOF LC-MS OF INTACT TRANSFERRIN (TRANSFERRIN GLYCOPROFILING)

As previously described, 5 μL plasma was immunoprecipitated with antitransferrin beads. Intact transferrin MS was performed on a microfluidics-based platform (Agilent Technologies) consisting of an Agilent 1260 nanoLC-HPLC-chip system using a C8 protein chip (C8-chip) coupled to an Agilent 6540 QTOF LC/MS system.[10] Data analysis for transferrin glycoprofiling was performed using Agilent Mass Hunter Qualitative Analysis Software B.05. The distribution of raw charge data was deconvoluted to reconstructed mass data using the Agilent BioConfirm Software.[10]

A set of 25 transferrin glycoforms (see Table II) was selected for relative quantitation, calculated to their total sum of abundance. These glycoforms were selected on the basis of their appearance in spectra of PGM1-CDG patients and consisted of complex, hybrid, and high mannose type N-glycans (see Fig 1A). The percentage of improvement (PI) of all glycan-indexes for treatment monitoring was calculated based on the following formula: $\left[\frac{(\text{index before treatment})-(\text{index after treatment})}{\text{high (LOGI \& LOCGI) or low (NGI) reference limit}}\right] \times 100$

HPLC-CHIP-QTOF LC-MS OF TOTAL PLASMA N-GLYCANS (TOTAL PLASMA GLYCOPROFILING)

As modified from previously described, 10 μL of plasma was mixed in equal parts with an aqueous solution of 200 mM ammonium bicarbonate and 10 mM dithiothreitol.[11] Protein denaturation was performed in mild conditions by alternating between boiling temperature and room temperature in a water bath for 6 cycles of 5 seconds each. For enzymatic release of N-glycans, 1 μL of peptide N-glycosidase F, PNGaseF (New England Biolabs, catalog no. P0704L) was added and the mixture was incubated for 22 hours at 37°C. To remove proteins from the glycans, ethanol precipitation was performed with 80% (volume/volume; v/v) ethanol, by adding 80 μL of ethanol and the mixture was frozen at −80°C for 45 minutes. The mixture was centrifuged at 14,000 rpm (Eppendorf) for 20 minutes and the supernatant was dried in vacuo (Thermo RVT4104 Refrigerated Vapor Trap). Purification and enrichment of glycans was performed using a graphitized carbon cartridge (Grace Davison, catalog no. G4240-64010, 150 mg, 4.0 mL). The cartridge was washed with 1.5 mL of 80% acetonitrile and 0.1% trifluoroacetic acid (v/v) in water, and conditioned with 3.0 mL of water. Dried samples were reconstituted with 200 μL of pure water before applied onto the cartridge, and then washed with 3.5 mL of pure water. Finally, plasma N-glycans were eluted with 2.0 mL of 40% acetonitrile and 0.05% trifluoroacetic acid (v/v) in water and dried in vacuo (Thermo RVT4104 Refrigerated Vapor Trap).

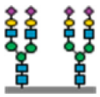
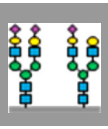
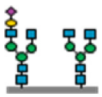
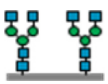
MS was performed on a microfluidics-based platform (Agilent Technologies) consisting of an Agilent 1260 Infinity HPLC-chip system using a porous graphitized carbon chip (PGC-chip) and Agilent 6540 QTOF mass spectrometer as described.[12] Raw LC-MS data were analyzed using the Molecular Feature Extraction algorithm included in Agilent Mass Hunter Qualitative Analysis Software B.05. Using a mass tolerance of 20 ppm, deconvoluted masses of each chromatogram peak were compared against a theoretical glycan mass library (in-house) consisting of all possible complex, hybrid, and high mannose type N-glycans that have been reported in human plasma.[12] Hence, only glycan compositions containing hexose (Hex), *N*-acetylhexosamine (HexNAc), fucose (Fuc), and *N*-acetylneuraminic acid (Neu5Ac) were included. Relative abundances of each glycan were obtained through normalization to the total signal of all detected glycans. Global degalactosylation, fucosylation, and sialylation indexes were calculated based on the total relative abundance of degalactosylated N-glycans, fucosylated N-glycans and sialylated N-glycans, respectively.

Table I. Overview of genetic and treatment data of 19 PGM1-CDG patients

Patient ID	Sex/age	Mutation data (cDNA)	(Protein)	Galactose therapy (clinical study) Treatment	Patient ID	References	Galactose therapy (for this glycan-indexes study) No. of samples analyzed	Remarks
P1	F/22	*c.988G>C*	*p.G330R*	Yes	P2	Tegtmeyer et al 2014[3]	-	-
		c.1129G>A	*p.E377K*		P3	Wong et al 2017[6]	2 plasma samples	before and upon therapy (3 months)
P2	F/22	*c.1507C>T*	*p.R503X*	Yes	P6	Tegtmeyer et al 2014[3]	-	-
		c.1507C>T	*p.R503X*		P8	Wong et al 2017[6]	2 plasma samples	before and upon therapy (3 months)
P3	M/46	*c.343A>G*	*p.T115A*	Yes	P1	Stojkovic et al 2009[1]	-	-
		c.1145-1G>C	*Splice*		P8	Tegtmeyer et al 2014[3]	-	-
P4	F/18	c.1264C>T	p.R422W	Yes	P1	Wong et al 2017[6]	N/A	-
		c.1588C>T	p.Q530X					
P5	M/14	*c.1010C>T*	*p.T337M*	Yes	P1	Ondruskova et al 2014[7]	-	-
		c.1508G>A	*p.R503Q*		P2	Wong et al 2017[6]	2 plasma samples	before and upon therapy (3 months)
P6	M/18	*c.361G>C*	*G121R*	No	P6	Wong et al 2016[8]	N/A	-
P7	M/18	*c.787G>T*	*p.D263Y*	No	-	-	-	-
		c.788A>G	*p.D263G*					
P8	F/21	*c.316T>C*	*p.Ile106Val*	No	-	-	-	-
P9	F/18	*c.871G>A/*	*p.Gly291Arg*	No	-	-	-	-
P10	F/6	*c.689G>A*	*p.Gly230Glu*	Yes	P6	Wong et al 2017[6]	2 plasma samples	before and upon therapy (3 months)
P11	M/4	*c.157_158delinsG*	*p.Gln53Glyfs*15*	Yes	P4	Wong et al 2017[6]	2 plasma samples	before and upon therapy (3 months)
		c.1507C>T	*p.Arg503**					
		c.661C>T	*p.Arg221Cys*					
		c.1258T>C	*p.Tyr420His*					
P12	M/56	*c.988G>C in exon 6*	*p.Gly330Arg*	Yes	P1	Voermans et al 2017[5]	6 plasma samples	before, upon (1, 3, and 6 months) & after therapy
		c.1258T>C in exon 8	*p.Tyr420His*					
		c.1264C>T in exon 8	*p.Arg422Trp*					
P13	M/7	*c.988G>C*	*p.Gly330Arg*	Yes	-	-	-	-
		c.1007C>G	*p.Pro336Arg*					
P14	F/1	c.661delinsC	p.Arg221Vfs*13	Yes	-	-	2 plasma samples	Before and upon therapy (3 months)
		c.988G>C	p.Gly330Arg					
P15	F/3	c.419G>A	p.Gly140Asp	Yes	-	-	2 plasma samples	Before and upon therapy (3 months)
		c.1597C>T	p.Arg533Trp					
P16	M/25	*c.1547T>C*	*p.L516P*	No	P1.2	Tegtmeyer et al 2014[3]	-	-
P17	M/17	*c.1547T>C*	*p.L516P*	No	P1.1	Tegtmeyer et al 2014[3]	-	-
P18	M/31	*c.1145-222G>T*	*p.G382Vfs*2*	No	P5.1	Tegtmeyer et al 2014[3]	-	-
P19	F/25	*c.1145-222G>T*	*p.G382Vfs*2*	No	P5.2	Tegtmeyer et al 2014[3]	-	-

cDNA, complementary DNA; ID, Identification; F, Female; M, Male; ; N/A, not available; P, Patient.

Table II. Transferrin glycoprofiling in PGM1-CDG and controls. Transferrin glycoforms are sorted based on their relative abundance in PGM1-CDG. Data represent the mean and 95% CI (in %) for the reference ranges. The glycans that do not show statistically significant differences in abundance (student's *t* test; $P \leq 0.05$) between normal control and PGM1-CDG are highlighted in gray

No.	Assigned structure	Glycoforms ID (Fig 1)	Glycoform composition (with intact transferrin)				Mass, Da	Transferrin glycoform	Reference ranges		*t* test
			Hex	HexNAc	dHex	Neu5Ac		Classification	Controls (n = 20)	PGM1-CDG (n = 19)	(*P* values)
1.		**25**	10	8	0	4	79,557	Complete N-glycans (normal glycoform)	92.5 (91.6–93.3)	43.7 (33.8–53.7)	1.9×10^{-12}
2.		**6**	5	4	0	2	77,351	Lack of 1 complete N-glycan (CDG-I glycoforms)	2.20 (1.80–2.60)	15.6 (13.4–17.9)	1.0×10^{-14}
3.		**2**	3	4	0	0	76,444	Galactose lacking N-glycans (CDG-I glycoforms)	0.06 (0.01–0.03)	9.76 (6.08–13.4)	2.4×10^{-6}
4		**1**	0	0	0	0	75,144	Lack of two complete N-glycans (CDG-I glycoforms)	0.08 (0.04–0.12)	5.48 (3.69–7.27)	2.1×10^{-7}
5		**4**	4	4	0	1	76,897	Galactose lacking N-glycans (CDG-I glycoforms)	0.02 (0.01–0.03)	4.76 (3.21–6.31)	1.6×10^{-7}
6		**24**	10	8	0	3	79,265	Sialic acid only lacking N-glycans (CDG-II glycoforms)	2.77 (2.24–3.30)	3.81 (2.66–4.97)	0.09
7		**11**	7	8	0	1	78,196	Galactose lacking N-glycans (CDG-II glycoforms)	0.22 (0.13–0.31)	2.48 (1.72–3.24)	3.5×10^{-7}
8		**8**	6	8	0	0	77,743	Galactose lacking N-glycans (CDG-II glycoforms)	0.14 (0.07–0.22)	2.06 (0.95–3.18)	9.3×10^{-4}

(*continued on next page*)

Table II (*Continued*)

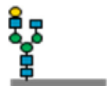
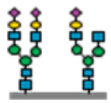
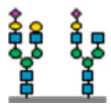
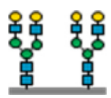
No.	Assigned structure	Glycoforms ID (Fig 1)	Glycoform composition (with intact transferrin) Hex	HexNAc	dHex	Neu5Ac	Mass, Da	Transferrin glycoform Classification	Reference ranges Controls (n = 20)	PGM1-CDG (n = 19)	*t* test (*P* values)
9		**16**	8	8	0	2	78,650	Galactose lacking N-glycans (CDG-II glycoforms)	0.12 (0.07–0.16)	1.72 (1.27–2.18)	7.4×10^{-9}
10		**3**	4	4	0	0	76,606	Galactose lacking N-glycans (CDG-I glycoforms)	0.04 (0.02–0.06)	1.43 (0.79 – 2.06)	5.0×10^{-5}
11		**5**	5	4	0	1	77,060	Sialic acid only lacking N-glycans (CDG-I glycoforms)	0.01 (0.00–0.01)	1.34 (0.95–1.74)	1.8×10^{-8}
12		**23**	9	8	0	3	79,103	Galactose lacking N-glycans (CDG-II glycoforms)	0.20 (0.13–0.27)	1.21 (0.87–1.55)	5.0×10^{-7}
13		**18**	9	8	0	2	78,809	Galactose lacking N-glycans (CDG-II glycoforms)	0.10 (0.05–0.14)	0.95 (0.73–1.17)	1.2×10^{-9}
14		**14**	10	8	0	0	78,399	Sialic acid only lacking N-glycans (CDG-II glycoforms)	0.15 (0.07–0.22)	0.82 (0.52–1.10)	3.4×10^{-5}
15		**10**	6	9	0	0	77,945	Galactose lacking N-glycans (CDG-II glycoforms)	0.06 (0.01–0.11)	0.75 (0.29–1.21)	3.1×10^{-3}

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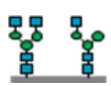
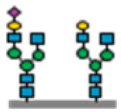
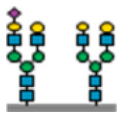
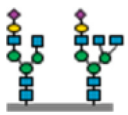
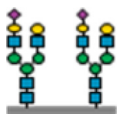
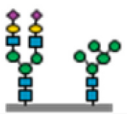
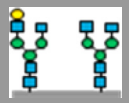
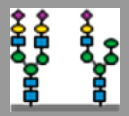
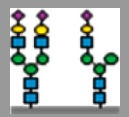
Table II (*Continued*)

No.	Assigned structure	Glycoforms ID (Fig 1)	Glycoform composition (with intact transferrin)				Mass, Da	Transferrin glycoform	Reference ranges		*t* test
			Hex	HexNAc	dHex	Neu5Ac		Classification	Controls (n = 20)	PGM1-CDG (n = 19)	(*P* values)
16		**12**	8	6	0	2	78,240	N-acetyl glucosamine only lacking N-glycans (CDG-II glycoforms)	0.20 (0.10–0.30)	0.66 (0.40–0.91)	1.4×10^{-3}
17		**7**	6	7	0	0	77,545	Galactose lacking N-glycans (CDG-II glycoforms)	0.20 (0.08–0.32)	0.64 (0.26 – 1.01)	2.4×10^{-2}
18		**13**	8	8	0	1	78,354	Galactose lacking N-glycans (CDG-II glycoforms)	0.32 (0.24–0.41)	0.63 (0.44–0.81)	3.4×10^{-3}
19		**17**	10	8	0	1	78,693	Sialic acid only lacking N-glycans (CDG-II glycoforms)	0.07 (0.03–0.10)	0.51 (0.23–0.78)	1.9×10^{-3}
20		**19**	8	9	0	2	78,853	Galactose lacking N-glycans (CDG-II glycoforms)	0.18 (0.11–0.24)	0.48 (0.29–0.67)	3.1×10^{-3}
21		**21**	10	8	0	2	78,971	Sialic acid only lacking N-glycans (CDG-II glycoforms)	0.12 (0.06–0.18)	0.40 (0.24–0.56)	1.3×10^{-3}
22		**15**	10	6	0	2	78,560	High mannose glycans (CDG-II glycoforms)	0.05 (0.01–0.08)	0.32 (0.15–0.49)	2.4×10^{-3}

(*continued on next page*)

Table II (*Continued*)

No.	Assigned structure	Glycoforms ID (Fig 1)	Glycoform composition (with intact transferrin) Hex	HexNAc	dHex	Neu5Ac	Mass, Da	Transferrin glycoform Classification	Reference ranges Controls (n = 20)	PGM1-CDG (n = 19)	*t* test (*P* values)
23		**9**	7	8	0	0	77,900	galactose lacking N-glycans (CDG-II glycoforms)	0.05 (0.02 – 0.08)	0.24 (0.01–0.50)	0.13
24		**22**	10	7	0	3	79,064	Hybrid glycans (CDG-II glycoforms)	0.21 (0.09–0.32)	0.13 (0.07–0.18)	0.19
25		**20**	9	7	0	3	78,900	N-acetyl glucosamine only lacking N-glycans (CDG-II glycoforms)	0.05 (0.00–0.11)	0.10 (0.04–0.15)	0.24

STATISTICAL ANALYSIS

Data were analyzed using GraphPad Prism (version 5.03) and IBM SPSS (version 22.0) software. Using a parametric approach, central 95% confidence intervals in the *control* group (n = 20) were used to express the reference limits. For the 3 selected transferrin glycoforms (glycoform 2, 4, and 6) and their prediction model, and also for the 6 global glycosylation indexes, student's *t* test was used for the specific comparison of glycans' relative abundance between PGM1-CDG and other groups (*control* and *contrast* groups). *P* values of ≤0.05 were considered significant. Backward (conditional) logistic regression of the glycoforms 2, 4, and 6 was performed using the SPSS to determine the equation variables (slope and y-intercept), for the calculation of the prediction model (linear regression) between PGM1-CDG and other groups (*control* and *contrast* groups). Receiver Operating Characteristic curve analysis was performed using the GraphPad Prism to calculate the diagnostic value (sensitivity and specificity), and the Area Under Curve (AUC) of the 3 combined transferrin glycoforms ($P < 0.0001$) for discriminating between PGM1-CDG and other groups (*control* and *contrast* groups).

RESULTS

PGM1-CDG in routine CDG screening. PGM1-CDG can present as a mixed CDG-I and CDG-II screening profile by TIEF, but has also been identified in CDG-I cohorts.[2] In our study of 19 patients (Fig 1 and Supplementary Table I), a mixed profile with elevated asialo- and disialotransferrin (CDG-I) was observed, in addition to elevated monosialo- and trisialotransferrin (CDG-II). Simultaneously, CDG screening in our PGM1-CDG cohort revealed patients with a more predominant CDG-I profile (Fig 1A) and others with mild CDG-II screening profile (Fig 1B), indistinguishable from other CDG-II defects. In view of the galactose lacking glycans in PGM1-CDG patients, we also performed CDG screening for mucin type O-glycosylation by Apo-CIII IEF in 9 patients (Supplementary Table I). No defect in mucin type O-glycosylation was observed, showing that PGM1-CDG most probably only affects protein N-glycosylation.

Glycomics profiling for the diagnosis of PGM1-CDG. To gain more insight into the exact structural changes, high-resolution QTOF MS of intact plasma transferrin was performed. Our study of 19 PGM1-CDG patients showed a broad range of MS spectra from severe PGM1-CDG profiles (eg, patient 11, P11) (Fig. 1, A) to mild PGM1-CDG profiles (eg, patient 3, P3) (Fig 1, B). The controls (Fig 1, C)

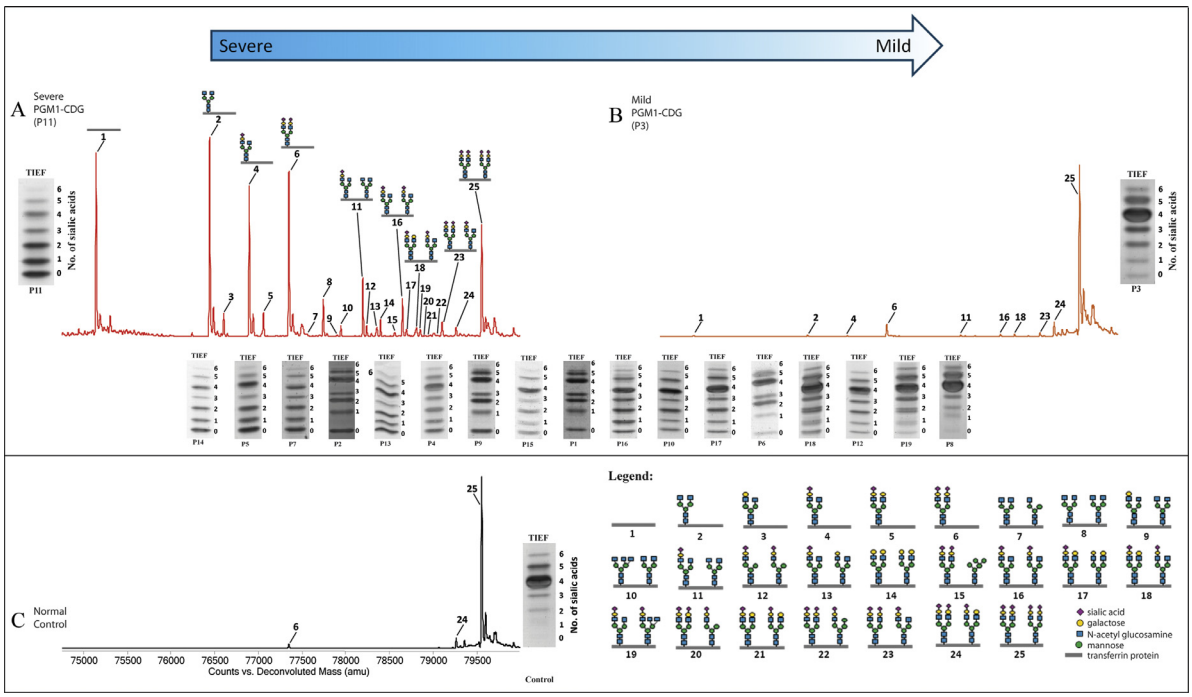


Fig 1. Heterogeneity of transferrin profiles in PGM1-CDG. (A) Severe or typical PGM1-CDG profiles as seen in P11 showed a clear mixed CDG-I/CDG-II pattern of TIEF. Deconvoluted mass spectrum of intact transferrin QTOF showed a high intensity of the peak indicating lack of complete glycans (glycoform 1 and 6) and a series of galactose lacking N-glycans (eg, glycoform 2, 4, 11, 16, 18, and 23). (B) Mild PGM1-CDG profiles as seen in P3 showed a mild CDG-II transferrin IEF. Deconvoluted mass spectrum of intact transferrin QTOF revealed a lack of 1 complete glycan (glycoform 6) as the highest abnormal peak as well as a series of typical PGM1-CDG degalactosylated glycoforms as also seen in severe PGM1-CDG profiles. (C) Deconvoluted mass spectrum of normal control profile and corresponding IEF showed a normal transferrin glycoforms (glycoform 25) as the most abundant transferrin glycoform in healthy subjects.

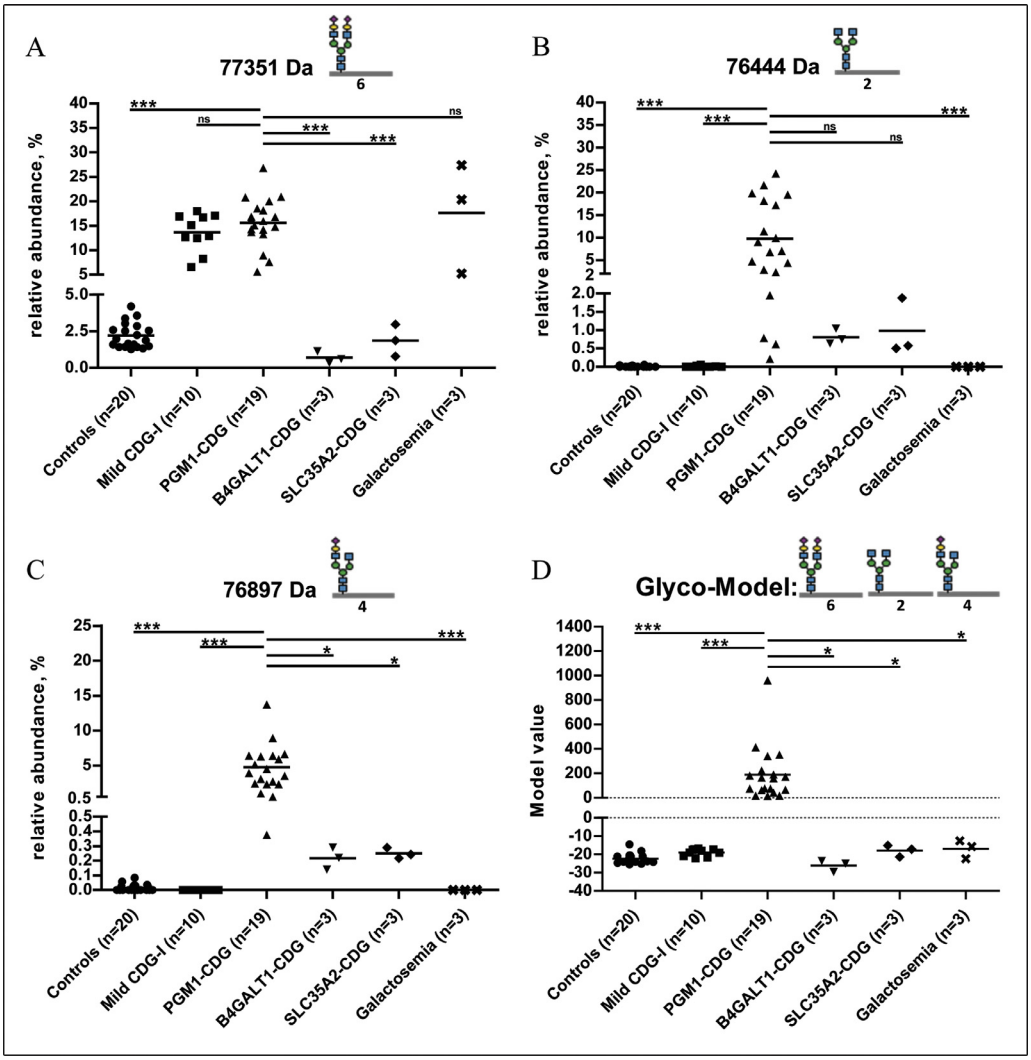


Fig 2. PGM1-CDG specific glycomarkers derived from glycoprofiling of intact transferrin. (A) Relative abundance of glycoform 6 (77,351 Da), the most abundant abnormal transferrin glycoform in PGM1-CDG. This CDG-I glycoform lacking 1 complete glycan, serves as a good marker to distinguish PGM1-CDG from CDG-II; (B) Relative abundance of glycoform 2 (76,444 Da) which is the most abundant degalactosylated glycoform in PGM1-CDG. This glycoform can also be seen in other galactose related CDG-II defects, such as B4GALT1-CDG and SLC35A2-CDG; (C) Relative abundance of glycoform 4 (76,897 Da), the only glycoform that allows discrimination of PGM1-CDG from other CDG group. However, this glycoform can't serve as a single glycomarker for PGM1-CDG because of its low abundance in some mild PGM1-CDG profiles; (D) The combination of 3 transferrin glycoforms (glycoform 6, 77,351 Da, glycoform 2, 76,444 Da, and glycoform 4, 76,897 Da) in a linear regression model showed a 100% discrimination of PGM1-CDG (n = 19) from normal controls (n = 20), mild CDG-I (n = 10), B4GALT1-CDG (n = 3), SLC35A2-CDG (n = 3) and galactosemia (n = 3).

showed 1 major peak that indicated the normal transferrin glycoform with 2 complete biantennary N-glycans (glycoform 25, 79,557 Da). In severe PGM1-CDG cases (Fig 1, A), dominant transferrin glycoforms of typical CDG-I were observed with a lack of 1 and 2 complete glycans (glycoform 6, 77,351 Da, and glycoform 1, 75,144 Da, respectively) and of mixed CDG-I and CDG-II with a lack of 1 complete glycan and galactose (glycoforms 2, 76,444 Da, and 4, 76,897 Da). Other CDG-II glycoforms were of lower abundance (glycoforms 3, 5, and 7-24). In mild PGM1-CDG profiles (Fig 1, B), the most abundant abnormal peak reflected a CDG-I glycoform lacking 1 complete glycan (glycoform 6; 77,351 Da) with only minor peaks corresponding to degalactosylated glycoforms. In contrast, TIEF profiles of this patient showed a mild CDG-II pattern (Fig 1, B), which complicates the diagnosis. However, both severe and mild PGM1-CDG MS profiles exhibited the same glycomics signature, namely the lack of complete glycans and galactose residues.

We then selected a comprehensive set of 25 transferrin glycoforms (Table II) for relative quantification and for discovery of specific glycomarkers for the unambiguous diagnosis of both mild and severely affected PGM1-CDG patients. We compared the relative

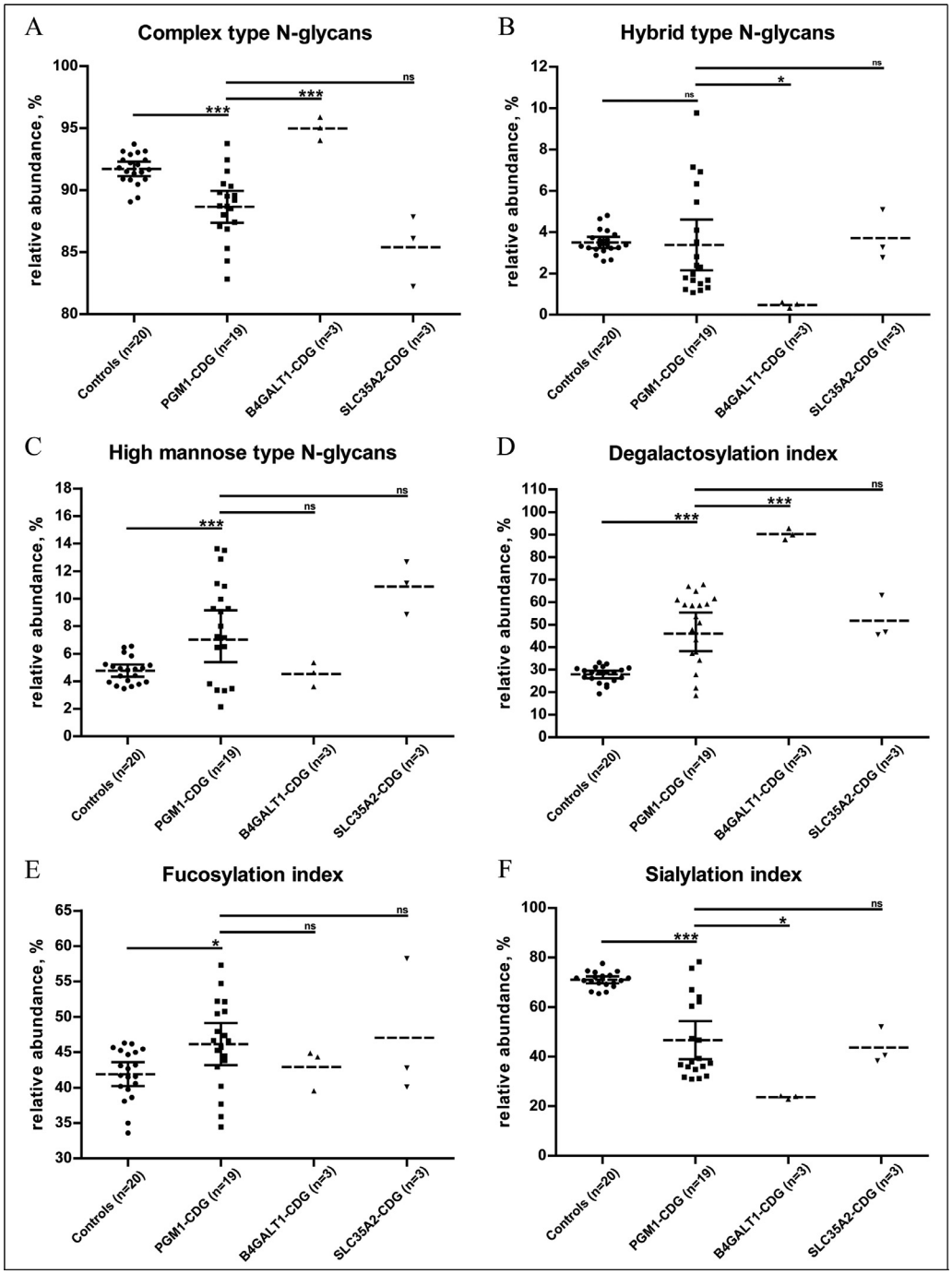


Fig 3. Total plasma glycoprofiling of the 6 glycan groups in PGM1-CDG. The relative abundance of 3 major classes of N-glycans, namely complex type N-glycans (A), hybrid type N-glycans, (B), and high mannose type N-glycans (C), in controls and PGM1-CDG patients. Global glycosylation indexes of degalactosylation (D), fucosylation (E), and sialylation (F), of the same subjects were also shown. In the majority of patients, complex glycans, hybrid glycans and sialylation index were reduced while high mannose glycans, degalactosylation index and fucosylation were increased as compared with controls. Data represent the mean and 95% CI (in %) for the reference ranges.

Table III. Total plasma N-glycans during D-galactose therapy.The 6 main glycan groups (Fig 3) before and after D-galactose treatment for P12. Data represent the mean and 95% CI (in %) for the reference ranges

Glycan types / indexes P12	Relative abundance, % Before treatment (month = 0)	After treatment (month = 6)	Reference ranges (n = 20)
Complex type glycans	88.8	91.7	91.7 (91.1–92.3)
Hybrid type glycans	2.0	3.0	3.5 (3.2–3.8)
High mannose type glycans	9.3	5.3	4.8 (4.3–5.2)
Degalactosylation index	58.8	34.7	27.9 (26.2–29.6)
Fucosylation index	46.7	43.6	41.9 (40.2–43.6)
Sialylation index	36.0	65.7	71.0 (69.6–72.4)

abundance of candidate glycans with controls, but also with potentially overlapping disease groups based on their glycomics profiling, including mild CDG-I and galactosemia (potentially mimicking mild PGM1-CDG) and 2 classes of CDG-II that are known to display galactose lacking glycans profile (B4GALT1-CDG and SLC35A2-CDG). The 2 most striking abnormal transferrin glycoforms in PGM1-CDG are glycoform 6 (77,351 Da) and glycoform 2 (76,444 Da). Glycoform 6 alone is a useful marker to differentiate PGM1-CDG from other CDG-II defects (eg, B4GALT1-CDG, etc), but is present at similar moderately elevated levels in mild CDG-I and galactosemia patients (Fig 2, A). Glycoform 2 is highly characteristic for PGM1-CDG, however, this glycoform was also present in other types of galactosylation defects such as B4GALT1-CDG and SLC35A2-CDG (Fig 2, B). Further analysis on the relative abundance of other glycoforms in all 5 disease-groups revealed that glycoform 4 (76,897 Da) was the only single transferrin isoform that was specific for PGM1-CDG. Due to its low abundance, this isoform cannot serve as a single marker for PGM1-CDG in some of the PGM1-CDG patients (Fig 2, C). We then combined all of these 3 transferrin glycoforms (glycoform 6, 77,351 Da; glycoform 2, 76,444 Da; and glycoform 4, 76,897 Da) and performed regression analysis to establish a prediction model for PGM1-CDG diagnosis. Finally, we showed that these 3 transferrin glycoforms are specific to PGM1-CDG (Fig 2, D) with 100% sensitivity and specificity in discriminating PGM1-CDG from normal controls (AUC = 1.0, $P < 0.0001$), mild CDG-I (AUC = 1.0, $P < 0.0001$), B4GALT1-CDG, SLC35A2-CDG, and galactosemia.

To investigate whether global N-glycosylation profiles can add value in PGM1-CDG diagnosis, we performed total plasma N-glycan profiling on all patients and 20 normal controls (Fig 3, and Supplementary Table 2). In addition, we also compared this glycomics profiling with B4GALT1-CDG and SLC35A2-CDG, which are known to show the high degree of global degalactosylation.[13,14] For the 3 major classes of N-glycans, the total relative abundance of complex type N-glycans (Fig 3, A) and hybrid type N-glycans (Fig 3, B) were reduced, while an increase was seen of high mannose type N-glycans (Fig 3, C) for the majority of patients. Further analysis on 3 global glycosylation indexes showed a very high degalactosylation index (Fig 3, D), which is in agreement with intact transferrin N-glycan profiles, an overall increase in the degree of fucosylation (Fig 3, E), and a reduction in sialylated N-glycans (Fig 3, F). As depicted from Figure 3, A–F, global galactosylation and sialylation were severely reduced in PGM1-CDG, B4GALT1-CDG, and SLC35A2-CDG, while global high mannose glycans and fucosylation were increased only in PGM1-CDG and SLC35A2-CDG.

Therapy monitoring in PGM1-CDG. Next, we aimed to explore the potential of glycoprofiling for monitoring the effectiveness of D-galactose supplementation in PGM1-CDG. After 6 months on oral D-galactose therapy, total plasma glycoprofiling in P12 showed an improvement of glycosylation in all 6 main glycan-indexes (Table III), indicating normalization of plasma protein N-glycosylation due to D-galactose supplementation. Improved addition of D-galactose to protein glycans after 6 months of D-galactose supplementation was seen from an increase of complex type N-glycans (from 88.8% to 91.7%), hybrid type-N-glycans (from 2.0% to 3.0%) and total sialylated N-glycans; sialylation index (from 36.0 to 65.7), and a decrease of high mannose type-N-glycans (from 9.3% to 5.3%) and total degalactosylated N-glycans; degalactosylation index (from 58.8 to 34.7). In addition, total plasma glycoprofiling showed an effect of D-galactose supplementation on the fucosylation level, with a decrease of total fucosylated N-glycans (from 46.7 to 43.6) to near normal. A similar improvement in global galactosylation, sialylation and fucosylation was observed in P1, P5, P14, and P15 after 3 months of D-galactose treatment, while fucosylation is maintained at a high level in P10 and increased in P2 and P11 (Supplementary Table III). Only P15 showed normalization of global galactosylation, sialylation, and fucosylation indexes.

Total plasma glycoprofiling is influenced by relative protein abundance and does not allow the detection of a lack of complete glycans (glycan loss), which is one of the main glycomics signatures for PGM1-CDG. Therefore, we defined 3 glycan-indexes from the transferrin QTOF spectra that are useful in clinical practice to monitor the therapeutic efficiency of dietary D-galactose in PGM1-CDG. As index to monitor the overall level of normalization, a Normal Glycan Index (NGI) was previously defined as the relative abundance of fully glycosylated transferrin (glycoform 25; 79,557 Da).[5] To discriminate the 2 main effects of PGM1 deficiency on glycosylation, we defined a Lack of Complete Glycan Index (LOCGI) and a Lack of Galactose Index (LOGI). For LOCGI, we used the relative abundance of glycoforms 1 and 6, lacking both and 1 glycan, respectively. For LOGI, we used the relative abundance of 6 degalactosylated glycoforms (glycoforms 2, 4, 11, 16, 18, and 23; See Table II and Supplementary Fig 1).

We used these glycan-indexes to study the effect of D-galactose therapy in 8 patients treated for 3 months. All patients showed an improvement of the transferrin glycoprofiles. For P12, the NGI showed near-normalization, indicating a good response to D-galactose treatment (Fig 4, A). The LOGI showed a fast response during D-galactose therapy (Fig 4, B), while the lack of complete glycans was restored more slowly, as displayed in the LOCGI (Fig 4, C). The percentage of improvement (PI) for 3 transferrin glycosylation indexes showed 82% normalization for NGI, 97% normalization for galactosylation (LOGI) and 73% normalization for the lack of complete glycans (LOCGI) after 6 months of D-galactose therapy. After discontinuation of D-galactose supplementation, the NGI, LOGI, and LOCGI worsened (Fig 4, A-C). The clinical and other biochemical improvements of P12 after 6 months galactose therapy have been reported in the previous study.[5] The TIEF and transferrin QTOF profiles of P12 before and upon galactose therapy are shown in Supplementary Figure 1.

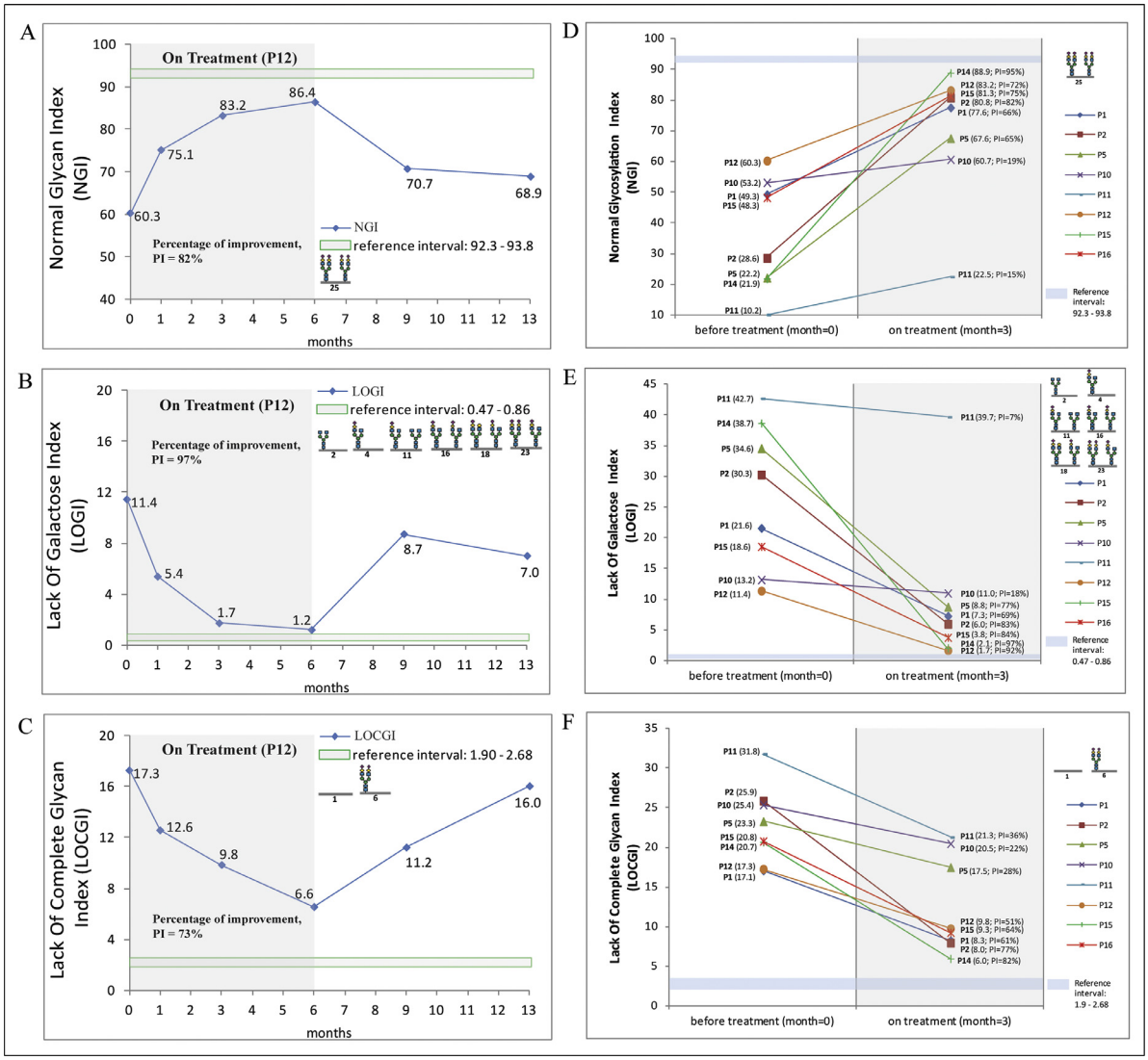


Fig 4. Transferrin glycoprofiling for therapy monitoring. The 3 glycan-indexes, (A) Normal Glycosylation Index (NGI), (B) Lack of Galactose Index (LOGI), and (C) Lack Of Complete Glycans Index (LOCGI), defined for therapy monitoring in PGM1-CDG were used to monitor the response of P12 before, upon and after 6 months D-galactose supplementation. The same transferrin indexes for (D) normal glycosylation (NGI), (E) galactose lacking glycans (LOGI), and (F) lack of complete glycans (LOCGI) are also shown on P1, P2, P5, P10, P11, P12, P14, and P15 to monitor the transferrin glycosylation improvements after 3 months of D-galactose therapy.

A similar improvement of the NGI (Fig 4, D), a fast recovery of the galactose index; LOGI (Fig 4, E) and slower recovery of the lack of complete glycans; LOCGI (Fig 4, F) was also observed in the majority of patients (P1, P2, P5, P12, P14, and P15) after 3 months of D-galactose therapy. Slow improvement of NGI, LOGI, and LOCGI was observed in P10 and P11, in whom the LOCGI restoration is more pronounced than LOGI.

DISCUSSION

The high clinical and biochemical heterogeneity in PGM1-CDG complicates diagnosis and necessitates the search for a specific marker for fast and accurate detection and evaluation of follow-up and treatment. In this study, we have solved the diagnostic challenge of PGM1-CDG by high resolution MS analysis of plasma transferrin. We here present a specific and reliable diagnostic test, which allows an automated way to diagnose PGM1 in large cohorts. The combination of 3 transferrin glycoforms was diagnostic for PGM1-CDG with high sensitivity and specificity. In addition, we defined 3 glycan-indexes to specifically monitor the normalization or improvement of transferrin glycoforms upon D-galactose supplementation.

Total plasma glycoprofiling showed an increase of both degalactosylated and fucosylated glycans. An increase of global fucosylation in the majority of PGM1-CDG patients has not been reported before. Since transferrin is a known protein with a low degree of fucosylation, this is an added value of total plasma glycomics. This novel finding also adds the PGM1-CDG to the subset of CDGs with high fucosylation profiles. Hyperfucosylation is associated with chronic hepatic inflammation, which can also be seen in PMM2-CDG (CDG-I) and B4GALT1-CDG.[13,15,16] However, neither fucosylation nor degalactosylation is specific for PGM1-CDG. Furthermore, the lack of complete glycans cannot be detected by total plasma glycoprofiling, which is the most important marker for sensitive detection of PGM1-CDG. Remarkably, an increase of global high mannose N-glycans in PGM1-CDG was also observed, similar to all patients with a UDP-galactose transporter defect (SLC35A2-CDG). Several cellular glycomics studies have shown an increase of high mannose glycans, particularly regarding Immnoglobulin G (IgG) glycosylation, attributed to: (1) a change in cellular nucleotide-sugar content (eg, UDP-galactose), which occurs in PGM1-CDG and SLC35A2-CDG;[17] (2) an increased osmolality and pH of Golgi and/or ER, which is common in CDG such as TMEM199-CDG;[9,18] (3) a low glucose concentration which is reflected by the hypoglycemia in PGM1-CDG;[19] and (4) an increased concentration of intracellular mannose and/or overactive GDP-mannose synthetic pathway which produce proteins with elevated levels of high mannose glycans.[20]

The high sensitivity and specificity of the combination of 3 transferrin glycoforms for PGM1-CDG underline the advantage of protein specific glycoprofiling for direct diagnosis of PGM1-CDG. The improvement over total plasma N-glycan analysis relates to: (1) the ionization of the glycoforms were dominated by the amino acid backbone, which improved relative quantification of glycans; (2) variations in protein concentrations were eliminated, which resulted in smaller reference ranges from healthy individuals. Also large population based serum glycomics studies show the interindividual glycome heterogeneity, which likely reflects the genetic, epigenetic, and metabolic systems.[21] The approach of intact protein glycoprofiling is attractive because of its robustness and fast analysis. This was also shown by a recent study that revealed the potential of intact haptoglobin glycoprofiling as a promising marker for cancer screening.[22] From a diagnostic point of view, the discovery of these glycomarkers has positioned transferrin QTOF MS as the primary test for PGM1-CDG with a turnaround time of only 2 hours.

Our previous study showed the biochemical improvement of D-galactose supplementation by transferrin glycoprofiling.[3,5,6] A specific and reliable diagnostic test is highly relevant since patients can present in a variety of clinical cohorts such as liver disease, exercise intolerance, or cardiomyopathy. Furthermore, early diagnosis and treatment might save the damage to muscles, liver, and heart. We introduced NGI, LOCGI, and LOGI from transferrin glycoprofiling to monitor D-galactose treatment and progression in PGM1-CDG. These indexes are more specific than the previous study especially to monitor the improvement of lack of galactose residues (LOGI) after galactose supplementation.[5,6] We showed for 6 patients that D-galactose therapy drastically improved the normalization of LOGI, which might easily be explained by the conversion of galactose to galactose-1-phosphate and subsequently to uridine diphosphate galactose, UDP-galactose. This can be rapidly used as a building block for protein glycosylation. Two patients showed a more pronounced improvement of the LOCGI than of the LOGI after 3 months of D-galactose therapy. It remains an open question how the D-galactose supplementation can improve the addition of glycans to proteins in the ER as shown by the improvement of the LOCGI. One next step is to relate the biochemical normalization of transferrin glycan-indexes (NGI, LOCGI, and LOGI) to the improvement of clinical features. This also offers an opportunity for clinicians to further optimize D-galactose supplementation (dosage, period, and alternative pharmacologic formulae) in order to achieve maximal biochemical and clinical effect.

In conclusion, transferrin glycoprofiling revealed a highly sensitive and specific glycomarker for the diagnosis of PGM1-CDG in its full clinical spectrum. In addition, 3 glycan-indexes are proposed, NGI, LOGI, and LOCGI, to monitor the effectiveness of D-galactose therapy in PGM1-CDG. This allows discriminating the response of endoplasmic reticulum versus Golgi glycosylation defect. We expect that these glycomarkers result in the diagnosis of additional PGM1-CDG patients and in guiding the further optimization of oral D-galactose as supplementation therapy.

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Conflicts of Interest: All authors have read the journal's policy on disclosure of potential conflicts of interest.

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SUPPLEMENTARY DATA

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